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AN ENZYME SEQUENCE TO DIGEST THE SOIL ORGANIC MATTER IN MULL AND MOR SOILS

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ABSTRACT

In researches connected with the mechanism of formation of mull and mor, an enzyme sequence has been developed which causes considerable release of the nitrogen contained in soil organic matter. In Nature, animal guts seem likely to contain not only a wide variety of enzymes, but the appropriate physiological conditions necessary to allow these enzymes to act on ingested organic matter.

INTRODUCTION

In the contrasting soil systems, mull and mor (Handley 1954), organic matter does not accumulate indefinitely. Despite this, attempts to digest soil organic matter, or the precursors of soil organic matter, enzymatically have not been particularly successful, Ladd and Butler 1967, Sowden 1970. This may be due to the rather drastic chemical procedures which have been used to extract organic matter from soil and result in material chemically unlike that occurring in Nature. The absorbtion and inactivation of enzymes, particularly by clays, organic colloids and phenolics, may also be important. The presence of proteins in soils is indicated by the production of amino acids by hot acid hydrolysis.

In connection with studies on mull and mor formation, a system has been developed to digest soil nitrogen. It differs

from previous work in that it uses a multitude of enzymes in sequence, with each enzyme being used under aerobic and anaerobic conditions. The sequence is considered an attempt to mimic gut conditions occurring in common soil animals.

MATERIALS AND METHODS

With the exception of worm and snail gut enzymes, enzymes were purchased from commercial sources. The quality and activity of enzyme batches varied appreciably when tested on appropriate substrates and invariably contained enzymes other than those stipulated. This latter finding was not considered a problem in the present work for in Nature, particularly in animal guts, it is likely that several enzymes will be operating in gut regions at any given time. The low molecular weight materials present in enzyme preparations (e.g. lactose, salts) were removed by a combination of dialysis and freeze drying. In the case of enzyme preparations containing cellulase fractional precipitations using ice cold acetone was substituted for the dialysis procedure to prevent enzymatic decomposition of dialysis bags. Worm and snail gut digestive juices were prepared using the procedure of Bawden and Pirie (1946). Approximately 100 large worms or snails were starved for 4 or 5 days to evacuate gut contents and the entire alimentary canals and associated digestive glands dissected out for the preparation of digestive juices.

Animals used included *Allolobophora* spp., *Lumbricus* spp. (earthworms) and *Helix* spp. (snails). Soils, leather, the remains of insects removed from spider webs and insectiverous plants, senescent leaf litters, and dead microbial and animal tissues were air dried and ground to fine powders. Table 1 indicates the range of materials which were used in the experiments, and as far as possible were collected in the field.

Duplicate amounts of these materials containing 1-10 mg total nitrogen were subjected to the enzyme sequence indicated (Fig.1). Each enzyme was used aerobically as follows. The enzyme, containing 0.1-1 mg total nitrogen (to give an enzyme : substrate N ratio of 1:10) was dissolved in calcium bicarbonate pH 5.5 buffer and centrifuged at 10,000 g for 10 min to remove any insoluble material and added to the substrate suspended in similar buffer containing ascorbic acid (reductant) and sodium stearate (surfactant), each at a concentration of one percent in a 50 ml polypropylene centrifuge tube. The total reaction volume was about 2-3 ml and on mixing with a glass rod gave a thick suspension. After the addition of 3-5 drops of chloroform, the tubes were capped and incubated at 6-10^{o}C for 2-4 days, with frequent stirring with a glass rod.

TABLE 1. MATERIALS USED IN ENZYME SEQUENCE EXPERIMENTS

WHOLE ANIMAL TISSUES (27 spp.)
centipedes, millipedes, mites, collembolans, beetles, flies, bugs, isopods, moths, butterflies, spiders, planarians, earthworms, slugs, snails, enchytraeids, webs, cocoons, nematodes, protozoans.

WHOLE MICROBIAL TISSUES (115 spp.)
bacteria, actinomycetes, algae, fungi (hyphae, rhizomorphs, mycorrhizae, sporophores)

WHOLE PLANT TISSUES (82 spp.)
Angiosperms, Gymnosperms, lichens, mosses, liverworts, horsetails, ferns, (leaves, roots, nodules, wood)

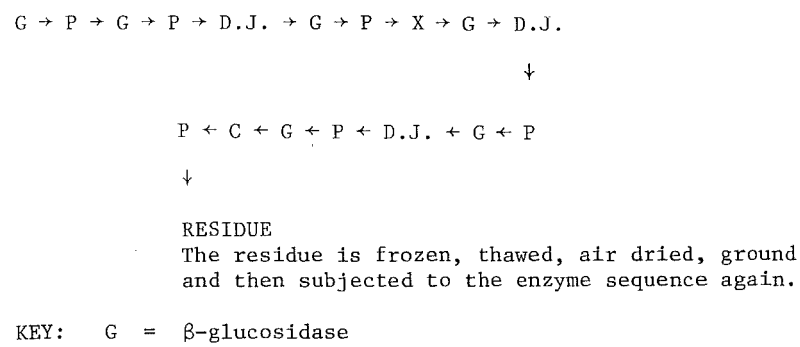
WHOLE SOILS (51 samples, 18 peats, 21 clays, 12 silt loams)
(forests, heaths, grasslands, arable)

LEATHER (9 samples 6 condensed, 3 hydrolysable)
ox and sheep skins tanned with hydrolysable and condensed tannins

CUTICLES (15 samples)
collected from spider webs and insectivorous plants

CELL WALLS (8 samples)
puffball, rhizomorph, bacterial and fungal cell walls

FIG. 1. ENZYME SEQUENCE



KEY:
G = β-glucosidase
P = pronase
D.J. = snail or worm digestive juice
X = mixture of cellulases, hemicellulases, pectinases and glucanases
C = chitinase

After each enzyme had been allowed to act the pH of the system was adjusted to 4 with 0.1M citric acid to reduce further enzyme action. The tubes were centrifuged for 10 min. at 10,000 g at 5°C and the supernatants decanted into Kjeldahl digestion-distillation flasks. The residues were twice washed with calcium bicarbonate buffer (prepared by bubbling CO_2 through lime water) and finally with the buffer that was to be used with fresh enzyme in the anaerobic sequence. Supernatants and washings were acidified and their total nitrogen contents determined by the Kjeldahl method.

The anaerobic system utilised similar reagents and, reaction volumes, in a sodium bicarbonate-disodium hydrogen phosphate pH 8 buffer (0.05M with respect to Na), as those used in the aerobic procedure. The tubes were flushed with CO_2 to provide anaerobic conditions before capping and placed in thick walled polythene bags or anaerobic jars which were flushed with CO_2 before sealing and incubation. At intervals during the incubation period (2-4 days at 6-10°C), the mixtures were shaken and the bags or jars flushed again with CO_2 to ensure that anaerobic conditions were maintained. At the end of the incubation period, the contents of each tube were acidified to pH 4 with 0.1M citric acid, centrifuged and washed as described above and prepared for the next enzyme in the sequence. In this way, each enzyme in the sequence was used firstly under aerobic and secondly under anaerobic conditions.

At the end of the entire sequence, any residue remaining was frozen, thawed, air-dried and ground in an attempt to simulate field conditions, which could prevail on animal excrements. This dried residue was then subjected to the entire enzyme sequence again.

Controls consisting of the reaction mixtures minus substrate, and reaction mixtures minus enzymes, were subjected to the same experimental conditions; any nitrogen dissolved was subtracted from the nitrogen determined in supernatants and washings derived from the complete reaction mixture. As a further check on the recovery of substrate nitrogen, any residues remaining at the end of the experiments were Kjeldahled and the nitrogen contained therein, plus the nitrogen dissolved as a result of enzyme action, was compared with the total nitrogen determined on untreated control substrates.

In other experiments, after determining nitrogen in an aliquot of supernatant, the remainder was used to provide the sole source of nitrogen for birch and tomato seedlings growing in non-sterile quartz sand plus nitrogen-free Hoaglands solution. Periodically, the entire plants were harvested and their nitrogen contents determined (after allowance for seed

nitrogen) to estimate how much of the enzymatically released nitrogen was actually taken up by the plants. The Kjeldahl method used involved use of a Hg catalyst, a salt-acid ratio of 0.5 and a digestion time of 3h. The recovery of nitrogen in standard solutions of ammonium sulphate, proteins and nicotinic acid by this procedure was quantitative and in the author's hands the method could reliably detect changes of nitrogen to at least 7 µg N.

The experiments described were begun in 1971 and are still in progress. They have been conducted in duplicate at least twice. In the case of bulked samples each consisting of mixtures of similar amounts of 18 animal spp.,48 microbial spp., 35 plant spp.,41 soils, 9 leathers, 15 cuticles and 8 cell walls they have been duplicated at least 6 times. Results between duplicates have been excellent (within 2% of each other) as have those obtained on aliquots taken from bulk samples and analysed at 2 yearly intervals.

RESULTS

In practice it was found that enzyme-N in control reaction mixtures minus substrate was completely soluble following citric acid addition and bicarbonate washes. This appears to be due to the fact that pH 4 is not a universal isoelectric point for all the enzymes used coupled with the property of calcium bicarbonate to dissolve all the enzymes used in the sequence. Pronase is an extremely aggressive enzyme and in preliminary experiments it was found that all the enzymes used in the sequence were extensively degraded as judged by the release of amino acids when incubated with pronase. It is likely that in the event of any enzyme material not dissolved by calcium bicarbonate it would have been digested by pronase since this enzyme was used alternatively in the enzyme sequence.

In the case of control reaction mixtures minus enzyme variable amounts of substrate-N were dissolved by the buffer and washing procedures. These amounts were determined for every substrate tested and on average amounted to 41% (range 9-52%) of the total substrate-N in the case of animal and microbial tissues. In the case of the remaining substrates 9% (range 2-15%) of the total substrate-N was soluble in control reaction mixtures minus enzymes. This naturally soluble substrate-N seems to be largely derived from cytoplasmic proteins and has, along with enzyme-N, been found to be mineralized under the experimental conditions described by Bremner (Bremner 1965).

The soluble nitrogen obtained in experimental reaction mixtures containing substrate plus enzyme after the complete enzyme sequence was derived from (1) enzyme-N, (2) naturally soluble substrate-N and (3) substrate-N released as a result of enzyme action. Since (1) and (2) were determined in control mixtures, addition of these two values and subtraction of this sum from the total soluble-N derived from experimental reaction mixtures containing substrate plus enzymes gave an estimate of (3) the nitrogen released from the substrate by enzyme action.

The total nitrogen in the residues remaining from control reaction mixtures minus enzymes was also determined. Addition of this value to that determined on the soluble-N occurring in these control mixtures gave a value which was within 1% of the total nitrogen value determined on untreated control substrates.

The total nitrogen in any residues which remained from experimental reaction mixtures containing substrate plus enzymes was also determined. These values added to those obtained by subtraction of control enzyme-N from the total soluble-N in experimental reaction mixtures, gave values within 1% of those determined on untreated control substrates. From these exercises it was concluded that no losses of nitrogen occurred and that the nitrogen released from experimental reaction mixtures after correction for enzyme and substrate-N controls actually represented substrate-N released by enzyme action. Further confirmation of this was obtained by comparing the weights (oven dry basis) of residues remaining from control reaction mixtures minus enzyme and experimental reaction mixtures consisting of substrate plus enzyme. After allowance for enzyme and naturally soluble substrate-N, considerable amounts (72-99%) of substrate-N were dissolved by the enzyme sequence and 60-70% of this nitrogen was taken up by plants (Table 2). The remainder of the dissolved nitrogen was probably incorporated into microbial biomass (as determined by direct counts and Jenkinson's fumigation procedure) which developed in these sand-plant systems. Tests using ^{15}N indicated that nitrogen fixation was negligible in the sand-plant systems. Animal, microbial and leather substrates were subjected to the enzyme sequence twice, whilst plant tissues required 5-6 exposures and soils 8-10 exposures to this enzyme sequence before considerable release of substrate nitrogen occurred.

Compared with other plant substrates the nitrogen contained in lichens was not particularly susceptible to enzyme attack approximately 64-71% of the total lichen-N was dissolved during the enzyme sequence. Most of the soils used in these experiments were selected on the basis of their high nitrogen contents, average 0.71% (range 0.42 - 1.31%). This made it considerably

TABLE 2. AVERAGE SUBSTRATE-N RELEASED BY ENZYMES (A), AND TAKEN UP BY PLANTS (B) RESULTS EXPRESSED AS A PERCENTAGE OF ORIGINAL SUBSTRATE-N

Material	System	Initial substrate weight (mg)	Final substrate weight (mg)	Initial substrate N (mg)	Residual substrate N (mg)	Substrate -soluble N (mg)	Enzyme N (mg)	A Substrate N released by enzyme as % of original substrate N	B % of original substrate N
Animal tissues	Control	79	53.2	6.79	4.28	2.51	0		
	Experimental	79	5.2	6.79	0.41	6.38	9.38	95 (83-100)	57
Microbial tissues	Control	150	78	5.95	3.21	2.74	0		
	Experimental	150	6.3	5.95	0.29	5.66	10.85	95 (89-100)	72
Plant tissues	Control	200	169	2.12	1.93	0.19	0		
	Experimental	200	9	2.12	0.15	1.97	3.88	93 (64-98)	65
Soils	Control	500	487	3.55	3.23	0.32	0		
	Experimental	500	439	3.55	0.81	2.74	5.56	72 (53-89)	62
Leathers	Control	50	44	5.43	5.22	0.21	0		
	Experimental	50	4.6	5.43	0.03	5.40	9.36	96 (98-100)	68
Cell walls and Cuticles	Control	100	77	5.73	5.00	0.73	0		
	Experimental	100	6.1	5.73	0.40	5.33	9.9	93 (75-100)	62

easier to follow changes in the amounts of N dissolved by enzymes. Peat and clay soils, whether originating from mull or mor sites did not show any marked differences in their susceptibility to enzyme attack. Nitrogen distribution analyses were performed on bulked quantities of soil residues remaining after exposure to the enzyme sequence and the forms of nitrogen found were qualitatively similar to those occurring in control residues. Quantitatively, with the exception of 'fixed-NH_4' which amount remained the same in treated and control residues, the amounts of these forms of nitrogen were lower in enzyme treated than control samples.

The appearance of soil and plant substrates following enzyme treatment was markedly different from that of control substrates. In both, considerable amounts of non-nitrogenous substances were removed and residues had a bleached appearance.

DISCUSSION

It should be stressed that the enzyme sequence described is only considered to approximate that occurring in Nature. In guts and excrements it is likely that a multitude of different enzymes simultaneously operate on organic matter and no doubt the type and activity of a particular enzyme will be influenced by the prevailing physiological conditions in particular gut regions. Attempts to develop a system in which all the enzymes were acting simultaneously were thwarted by the fact that pronase digested the other enzymes. The substitution of pronase by pancreatin or trypsin somewhat alleviated this problem and it may be that systems of this type occur in Nature for the nitrogen and other nutrients in organic matter are slowly but continuously released over a considerable period of time.

In this preliminary report, it is not possible to discuss extensively the mechanisms of enzyme action and the subsequent release of nitrogen even if these were adequately known, but attention should be given to Handley's paper (1961 and references therein) in which he correlates and begins discussion on various aspects of the enzymatic digestion of leather. In the present work, complete dissolution of finely ground leather could be achieved solely by the alternate use of β-glucosidase and collagenase. This appears to be understandable on the following grounds. In Nature the tannins and their precursors are linked to carbohydrates. During the oxidative tanning of collagen in vitro or of plant proteins during leaf senescence, amino groups in the protein, probably other than those involved in peptide

linkage, react with quinone groups derived from tannin hydroxyl groups. It is probable that the tannins themselves undergo further polymerization so that the protein molecule is penetrated and surrounded by tannins linked to amino groups, sugar groups and other tannins, (Ladd and Butler 1975). Although the mechanisms of these reactions are not fully understood the resultant complex is resistant to proteolytic enzymes. This appears to be due to steric hindrance effects and inactivation of enzymes by quinone groups, this latter effect could be regarded as another extension of the tanning reaction. Field observations suggest that leaf litter, which contains tanned proteins physically and perhaps covalently attached to plant cell wall carbohydrates, does not accumulate indefinitely. Theoretical considerations indicated that any enzyme involved in the degradation of ground leaf litter or leather must be capable of not only overcoming steric hindrance effects but also the well known inactivating effects of phenolics and tannins, (Loomis and Battaile 1966, Goldstein and Swain 1965, Harborne 1979, Swain 1979). In the older literature it was noted that glucosidases were often obtained by tannin precipitation (Stone 1961) and it was argued that the tannin sugar bonds could be broken using glucosidases thus providing "chinks in the armour" in tanned substrates. This would perhaps expose regions of the protein to proteolytic enzymes but in order for these to act precautions were considered necessary to prevent regeneration of reactive groups e.g. quinones and absorption effects due perhaps to hydrogen bonding. From an examination of animal guts this was attempted by the use of reductants such as ascorbic acid in conjunction with a reducing atmosphere of CO_2. The use of sodium stearate reduced the tendency for absorption effects. As far as was technically possible attempts were made to recreate physiological conditions likely to occur in Nature. The use of surfactants such as Triton, reductants such as thioglycollic acid, temperatures of the order of 40-50^{o}C and largely aqueous reaction environments were considered not only unrealistic of the natural conditions but possible of artifact formation if used in reaction mixtures. It may prove profitable to use leather in future studies to understand the mechanisms of the reactions involved in N release, from tanned substrates, because leather comprises proteins, carbohydrates and tannins. Tanned materials of this type occur universally in Nature and demonstrate similar resistance to proteolytic enzymes at least.

The results in this paper suggest that the enzyme sequence technique, if used in conjunction with techniques based on the principles of chromatography, spectrometry and nuclear magnetic resonance, could provide a useful tool with which to study the nature of soil organic matter.

In any case, the results are compatible with the field observations of the characteristic biological differences which occur in mull and mor systems, and have been briefly discussed by Greenfield (1981). In the classic situation of adjacent mull and mor supporting beech, the greater quantities of animals such as earthworms and snails are present in the mull: this ensures that much of the year's litterfall (as such and in excrements) would be passed through the guts of these animals many times and hence subjected to enzyme sequences and physiological conditions of the types mentioned in this paper. The numbers and kinds of animals present in mull will probably be determined by many factors, but the system in the absence of man will have developed a natural level of animal biomass, and it is this which is likely to be of major importance in determining how much organic matter, visibly identifiable or comminuted, is ingested each year. The gut enzymes are probably of microbial and animal origin, but it is important to realise that guts, and to some extent excrements, contain the necessary physiological conditions for prolonged enzyme action.

In the beech mor, there are few earthworms and those that are present are normally of a type adapted to living in the acidic humus layer. The animal biomass, and hence guts, are but a fraction of those which occur in the beech mull. Consequently, only small quantities of the year's litter will be subjected to prolonged or sustained gut enzymes. In this system, in the comparative absence of guts, it is probable that litter will be subjected to microbial decomposition; field observation supports this view. From an enzymatic viewpoint, decomposition is likely to be incomplete or follow a different course because it is difficult temporally and spatially to picture where conditions in such an acidic milieu could be as optimal as those occurring in animal guts.

Even if optimal sites do occur on microbial surfaces, they must be in small number as judged in the final outcome by the lesser amount of plant tissue production on beech mor than on adjacent beech mull. This incomplete microbial decomposition results in the production of large amounts of organic acids (derived from carbohydrates) which, due to the lack of bases especially calcium, are probably largely leached away and, in the long term, are an important cause of the bleached horizons which characterise mor systems. Acidic substances which are produced during enzyme action in anaerobic gut regions are likely to be metabolized further in other gut regions. Due to the presence of available bases in guts and excrements, particularly in mull soils, these acids will also be neutralised prior to further decomposition. These conditions favour large numbers of earthworms and may help to explain why mull soils are characteristically well mixed and have no bleached horizons.

Mull and mor systems containing senescent plant, animal and microbial tissues occur world wide and, in the main, the nitrogen in these tissues is tanned. Such tanned complexes vary in resistance to enzymic attack, depending on whether the system is mull or mor. These differences have been ascribed in the first instance to differences in the biology of both systems (Muller 1884). The animals in mull differ qualitatively and quantitatively from those in mor, and it is this observation which has lead not only to the enzyme sequence in this paper, but has strengthened the view that the concept of mull and mor is a fundamental unifying concept in soil biology and that the fertility of a particular soil is a function principally of the biology of that soil.

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